The Effects of 6-Chloro-8-aza-9-cyclopentylpurine on Nucleic Acid and Protein Synthesis in *Escherichia coli*
II. In Vitro Studies

JAMES M. JOHNSON AND RAYMOND W. RUDDON[1]

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48104

MORRIS S. ZEDECK[2] AND ALAN C. SARTORELLI

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

(Received February 7, 1969)

SUMMARY

The purine deoxyribonucleoside analogue 6-chloro-8-aza-9-cyclopentylpurine has been found to inhibit DNA, RNA, and protein synthesis independently in *Escherichia coli*. The mechanism by which it inhibits DNA synthesis apparently involves a site of action in the pathway for the formation of thymine nucleotides, since the conversion of uracil or cytosine to thymine nucleotide derivatives in whole cells was completely abolished by 0.5 mM 6-chloro-8-aza-9-cyclopentylpurine within minutes after addition of drug to a growing culture. *In vitro* studies demonstrated that concentrations of the analogue as high as 2 mM had no effect on thymidylate synthetase activity; therefore, blockade of thymine nucleotide formation is apparently produced at an earlier step. The conversion of uracil or cytosine to nucleotide derivatives other than thymine derivatives was decreased 50–60% by 6-chloro-8-aza-9-cyclopentylpurine (0.5 mM) in whole cells, suggesting that inhibition of the formation of ribonucleotides contributes to the blockade of both DNA and RNA synthesis observed *in vivo*. Preliminary incubation of partially purified polymerase preparations with the analogue (1 mM) resulted in marked inhibition of RNA polymerase activity, with only slight effects on DNA polymerase. The template function of purified DNA, however, was not affected by incubation of DNA with drug for up to 16 hr prior to assay of template activity. Inhibition of protein formation was also observed after incubation of components of the cell-free protein-synthesizing system *in vitro*. The effect of the drug on protein synthesis appeared to be due to an action on the 100,000 × *g* supernatant enzymes involved in the polymerization of amino acids into polypeptide linkage. Neither aminoacyl-tRNA synthetase activity nor the ability of the ribosomes to support protein synthesis was affected by 6-chloro-8-aza-9-cyclopentylpurine. The inhibitory effects of the drug on the activity of enzymes involved in the formation of nucleic acids and protein observed *in vitro* required prior incubation with the drug and were irreversible. The data suggest that a portion of the actions of 6-chloro-8-aza-9-cyclopentylpurine are due to a site-directed alkylation of enzymes, leading to a blockade of enzymatic activity.

This study was supported by a grant from the Connecticut Division of the American Cancer Society, and by Grants CA-02817, 5-T4-CA-5012, and CA-02992 from the United States Public Health Service.

[1] To whom requests for reprints should be addressed.

[2] Present address: Division of Pharmacology, Sloan-Kettering Institute for Cancer Research, New York, New York 10021.

INTRODUCTION

The purine deoxyribonucleoside analogue 6-chloro-8-aza-9-cyclopentylpurine has been shown to inhibit cellular growth, the synthesis of induced enzymes, and the formation of DNA, RNA, and protein in two microorganisms, *Escherichia coli* and *Pseudomonas testosteroni* (1–3). The preceding paper in this series (3) examined the effects of this agent on *E. coli* B and showed that in whole cells 689[3] (0.4 mM) produced immediate and essentially complete inhibition of DNA synthesis; the formation of RNA and protein was also blocked approximately 70% by this agent. This report (3) also provided evidence that the inhibition of the syntheses of DNA, RNA, and protein by this agent was in part the result of separate metabolic lesions induced by the drug. In the present paper, the effects of 689 on the formation of protein and nucleic acids were studied in cell-free systems derived from *E. coli* B cells in order to delineate the critical subcellular sites of action of 689. The purine deoxyribonucleoside analogue was observed to react with and irreversibly inhibit several enzymes involved in the fabrication of nucleic acids and protein.

MATERIALS AND METHODS

Preparation of cell fractions from E. coli B. *E. coli* B cells were grown at 37° in either a glucose-enriched Difco broth (4) or a minimal salts medium (5) and harvested in the early logarithmic phase of growth. The cells were disrupted and extracted, and S-30, ribosomal, and S-100 fractions were prepared by the method of Nirenberg and Matthaei (6). The protein content of all fractions was determined by a modification of the method of Lowry *et al.* (7).

In some cases, the ribosomal suspension (approximately 14 absorbance units at 260 mμ) was layered on top of a 4.6-ml linear sucrose gradient (5–20%) containing 10 mM Tris-HCl (pH 7.8), 10 mM magnesium acetate, and 60 mM KCl, and centrifuged for 2 hr at 35,000 rpm in a Spinco SW 50L rotor. Five-drop fractions were collected, and the absorbance at 260 mμ was determined.

To dissociate the 70 S particles into 50 S and 30 S subunits, the ribosomal pellet was suspended in 10 mM Tris-HCl buffer, pH 7.8, containing 0.1 mM magnesium acetate, 60 mM KCl, and 6 mM β-mercaptoethanol. The suspension was heated at 45° for 30 min, placed on a sucrose gradient, and centrifuged as above.

A deoxyribonucleoprotein preparation was isolated from the S-100 fraction by differential and density gradient centrifugation as described by Shin and Moldave (8). The fraction contained 362 and 159 μg of protein and DNA per milliliter as assayed by the Lowry method (7) and diphenylamine reaction (9), respectively. The preparation contained both DNA polymerase (10) and RNA polymerase activity (8).

Preparation of a cell membrane fraction from E. coli B. A cell membrane fraction was prepared from *E. coli* B cells by a modification of the method described by Crouch (11). A 250-ml culture of *E. coli* B cells in a minimal salts medium (5) was vigorously shaken until the absorbance of the culture was 0.65 at 650 mμ. The culture was then centrifuged, and the cell pellet was resuspended at 25° in 5 ml of 10 mM Tris-HCl buffer, pH 7.9, containing 1 mM β-mercaptoethanol and 5 mM EDTA. To this suspension were added 15 ml of a 50% solution of sucrose in the above buffer, and lysozyme to a concentration of 0.5 mg/ml. The mixture was incubated at 37° for 40 min and centrifuged at 10,000 rpm in a refrigerated Sorvall centrifuge. Light microscopy showed the presence of protoplasts in the pellet. The protoplast pellet was then lysed by resuspension at 5° in 25 ml of 10 mM Tris-HCl, pH 7.9, containing 1 mM β-mercaptoethanol, using a wide-bore, 5-ml pipette. The resuspension process was continued for 20 min, when the mixture became very viscous. The suspension was centrifuged at 35,000 × *g* for 20 min; the

[3] The abbreviations used are: 689, 6-chloro-8-aza-9-cyclopentylpurine; poly U, poly A, and poly C, polyuridylic, -adenylic, and -cytidylic acids, respectively; DMSO, dimethyl sulfoxide.

pellet was resuspended as described above, and recentrifuged. The resulting pellet was finally suspended in 10 ml of the above buffer. This suspension of membranes had a protein content of 2 mg/ml. The membrane particle isolated by this method appears to be circular in shape and contains DNA, RNA polymerase activity, and DNA polymerase activity. The preparation incorporated nucleoside triphosphates but not nucleosides into DNA and RNA, indicating the negligible presence of intact cells.

Source of enzymes. DNA polymerase (EC 2.7.7.7), fraction IV, was prepared from late log phase *E. coli* B (purchased from General Biochemicals) according to the method of Richardson *et al.* (12). Fraction IV contained 4.5 mg of protein per milliliter and had a specific activity of 159 units/mg of protein. The enzyme was stored at −60° and showed no loss of activity for 9 months.

RNA polymerase (EC 2.7.7.6), fraction 4, was also prepared from commercially grown *E. coli* B cells as described by Chamberlin and Berg (13). The enzyme preparation had a protein content of approximately 1 mg/ml and a specific activity of 487 units/mg of protein. The enzyme was stored at 0–2° and lost 80% of its activity within 1 month.

Polynucleotide phosphorylase (EC 2.7.7.8) was purified from commercial *E. coli* B cells through the initial four steps described by Williams and Grunberg-Manago (14). The preparation had a protein content of 55 mg/ml and a specific activity of 20.2 units/mg of protein.

E. coli B thymidylate synthetase containing 8.5 units of activity per milligram of protein was a gift from Dr. M. I. Smith Lomax (15). *E. coli* alkaline phosphatase (EC 3.1.3.1), bovine pancreatic deoxyribonuclease (EC 3.1.4.5), ribonuclease (EC 2.7.7.16), trypsin (EC 3.4.4.4), and calf thymus DNA were purchased from Worthington Biochemical Corporation.

Enzyme assays. Purified RNA polymerase was assayed by the method of Chamberlin and Berg (13). RNA polymerase activity in the deoxyribonucleoprotein complex and cell membrane preparation was measured by the method of Shin and Moldave (8). Purified DNA polymerase was assayed as described by Richardson *et al.* (12). The activity of DNA polymerase in the deoxyribonucleoprotein complex and in the cell membrane preparation was measured in the manner described for RNA polymerase except that deoxyribonucleoside triphosphates were utilized as precursors. In all the assay procedures described above, the incorporation of ^{14}C-labeled nucleoside triphosphate into nucleic acid was measured using Millipore filtration (10).

Polynucleotide phosphorylase, thymidylate synthetase, deoxyribonuclease, ribonuclease, trypsin, and alkaline phosphatase were assayed for activity by the methods of Williams and Grunberg-Manago (14), Smith Lomax and Greenberg (15), Kunitz (16), Kalnitsky *et al.* (17), Hummel (18), and Garen and Levinthal (19), respectively.

Amino acid incorporation studies. The basic incubation mixture for measurement of the rate of incorporation of amino acids into protein by S-30, S-100, and washed ribosomal fractions was that described previously (20), except that the radioactivity incorporated into protein was determined by Millipore filtration in the present experiments. In some cases either poly U (25 μg), poly A (25 μg), or poly C (100 μg) was employed as exogenous messenger RNA to determine the rate of incorporation of phenylalanine, lysine, and proline, respectively, into polypeptide.

Assay of ^{3}H-poly U binding to ribosomes. ^{3}H-Poly U (0.5–1.0 μC; 22–27 mC/mmole) was added to a ribosomal suspension and incubated at 0° for 30 min (21). The mixture was then layered on a sucrose gradient and centrifuged as described above. Five-drop fractions were collected and assayed both for absorbance at 260 mμ and for radioactivity.

Assay of aminoacyl-tRNA synthetases. The activity of these enzymes in the S-100 fraction was determined by measuring the binding of ^{14}C-amino acids to tRNA using the following reaction mixture: 500 μg of S-100 protein, 200 μg of *E. coli* B stripped sRNA, 0.8 μmole of ATP, 5 μmoles of

sodium phosphoenolpyruvate, 10 μg of pyruvate kinase, 25 μmoles of Tris-HCl (pH 7.2), 2 μmoles of β-mercaptoethanol, 35 mμmoles each of 19 nonradioactive amino acids, and 0.12 μC of ^{14}C-amino acid in a total volume of 0.4 ml. The reactions were incubated at 37° for 12 min and stopped with 5% trichloracetic acid. The product was isolated by the technique of Millipore filtration, and radioactivity was measured in a scintillation spectrometer. As controls, some of the mixtures were heated in 5% trichloracetic acid at 90° for 20 min before Millipore filtration; this process solubilized more than 97% of the radioactivity, demonstrating that the major portion of the labeled amino acid was not bound to protein, but to tRNA.

Determination of the biosynthesis of nucleic acid precursors in vivo. Uracil-2-^{14}C (10 μg; 5 μC) was added to 5 ml of a logarithmically growing culture of *E. coli* B cells in minimal salts medium (5). After 3 min, the cells were harvested and washed once with ice-cold fresh medium. The cell pellet was extracted with cold, 0.4 N $HClO_4$. The mixture was centrifuged, and the acid-soluble fraction was neutralized with 8 N KOH. The insoluble $KClO_4$ was removed, and the supernatant was evaporated to dryness. The residue was dissolved in a small quantity of water, and the entire solution was applied to Whatman No. 1 filter paper and subjected to descending chromatography using a mixture of 1-butanol–glacial acetic acid–H_2O (10:1:3) (v/v) as the solvent. All nucleotide forms remained at the origin, while unconverted uracil-2-^{14}C migrated down the paper. The nucleotides were eluted from the origin with distilled water, and the eluate was incubated for 60 min at 30° in a mixture containing 50 mM Tris-HCl (pH 9.0), 60 mM $MgSO_4$, and 1 mg of lyophilized rattlesnake venom per milliliter. The reaction was stopped with cold acetone–glacial acetic acid (9:1) and centrifuged. The resulting nucleosides were then separated by paper chromatography utilizing two separate, descending systems with (a) 1-butanol–glacial acetic acid–H_2O (10:1:3) (v/v) and (b) isobutyric acid–15 N NH_4OH–H_2O (66:1:33) (v/v). The areas of the chromatogram corresponding to uridine, cytidine, deoxycytidine, thymidine, and deoxyuridine were located using nonradioactive markers, and the radioactivity of each nucleoside was determined.

Source and preparation of 689. 6-Chloro-8-aza-9-cyclopentylpurine was prepared as previously described (1). The material was dissolved in dimethyl sulfoxide, and the solution was diluted with H_2O until the final concentration of DMSO was 50%. All further dilutions of the drug solution were made with 50% DMSO.

Preliminary incubation of enzymes and other factors with 689. DNA polymerase (10 μg) was incubated at 37° with 689 in 0.2 ml of reaction mixture containing 12.5% DMSO, 50 mM Tris-HCl (pH 7.5), 25 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, and 25 μg of bovine serum albumin. RNA polymerase (25–50 μg) was incubated at 37° with 689 in 0.2 ml of reaction mixture containing 12.5% DMSO, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 2.5 mM β-mercaptoethanol, and 25 μg of bovine serum albumin. Polynucleotide phosphorylase (27 μg) was incubated with 689 at 37° in 0.2 ml of reaction mixture containing 12.5% DMSO, 50 mM Tris-HCl (pH 7.5), and 7.5 mM $MgCl_2$. Deoxyribonuclease, ribonuclease, and alkaline phosphatase (10 μg each) were incubated with 689 at 37° in 0.2 ml of reaction mixtures containing 12.5% DMSO and 15 mM Tris-HCl, pH 7.5. Trypsin (20 μg) was incubated with 689 at 25° and 37° in 0.2 ml of a reaction mixture containing 12.5% DMSO and 15 mM Tris-HCl, pH 7.5. Deoxyribonucleoprotein (104 μg of protein and 31 μg of DNA) was incubated at 37° with 689 in 0.2 ml of a reaction mixture containing 17% DMSO, 15 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl, and 0.7 mM β-mercaptoethanol. DNA (30 μg) was incubated with 689 at 37° in 0.2 ml of a reaction mixture containing 12.5% DMSO and 15 mM Tris-HCl, pH 7.5. Fractions S-30 and S-100 (500–800 μg of protein) were incubated at 37° in 0.2 ml of

reaction mixture containing 12.5% DMSO, 15 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 30 mM KCl, and 3 mM β-mercaptoethanol. Thymidylate synthetase (20 μg) was incubated with 689 at 25° in 0.2 ml of reaction mixture containing 12.5% DMSO, 30 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 5 mM β-mercaptoethanol. The membrane preparation (200 μg of protein) was incubated with 689 at 37° in 0.2 ml of reaction mixture containing 12.5% DMSO, 15 mM Tris-HCl, pH 7.5, and 0.5 mM β-mercaptoethanol. Washed ribosomes (300–500 μg of protein) were incubated with 689 at 37° in 0.2 ml of reaction mixture containing 12.5% DMSO, 7.5 mM Tris-HCl (pH 7.5), 7.5 mM magnesium acetate, 45 mM KCl, and 4.5 mM β-mercaptoethanol. For each of these incubations, control reaction mixtures, which contained all the constituents listed for the particular mixture, were incubated in the absence of drug.

Source of chemicals. ATP, GTP, CTP, UTP, TTP, dCTP, dATP, and dGTP were products of P-L Biochemicals; uridine 5′-triphosphate-2-^{14}C (24.9 μC/μmole), uniformly labeled adenosine 5′-triphosphate-^{14}C (425 μC/μmole), uniformly labeled cytidine 5′-triphosphate-^{14}C (331 μC/μmole), thymidine 5′-triphosphate-2-^{14}C (30.3 μC/μmole), and uracil-2-^{14}C (56 μC/μmole), New England Nuclear Corporation; sodium phosphoenolpyruvate, pyruvate kinase, sodium tetrahydrofolate, amino acids, and ADP, Calbiochem; L-asparagine and L-glutamine, Nutritional Biochemicals; adenosine 5′-diphosphate-8-^{14}C (38.6 μC/μmole) and deoxyuridine 5′-monophosphate-5-^{3}H (10 mC/μmole), Schwarz BioResearch, Inc.; polylysine and stripped *E. coli* B sRNA, General Biochemicals; sodium *p*-nitrophenyl phosphate and dUMP, Sigma Chemical Company; L-phenylalanine-^{14}C (15.8 μC/μmole), L-valine-^{14}C (6.9 μC/μmole), L-leucine-^{14}C (7.9 μC/μmole), L-proline-^{14}C (30 μC/μmole), L-lysine-^{14}C (7.5 μC/μmole), and L-serine-^{14}C (7.5 μC/μmole), all uniformly labeled, Nuclear-Chicago; poly U, poly C, and poly A, Miles Chemical Company.

RESULTS

Inhibition of RNA and DNA polymerase activity by 689. DNA, RNA, and induced enzyme synthesis have been observed to be strongly inhibited in *E. coli* B by treatment with 0.4 mM 689 (3). Moreover, the replication of T2osr bacteriophage was also inhibited in cells which had been treated with 689 for 15 min prior to infection (drug was removed from the medium before infection). These findings suggested that the purine deoxyribonucleoside analogue may function to inhibit enzymes involved in the synthesis of nucleic acids.

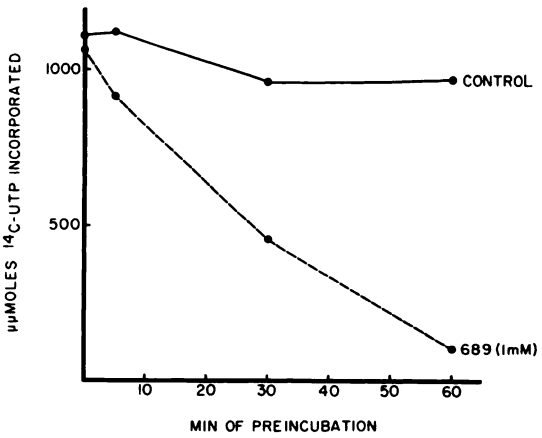

FIG. 1. *Effect of 689 on the activity of RNA polymerase*

RNA polymerase was incubated with 689 for various periods of time and then assayed for activity as described in MATERIALS AND METHODS.

The effect of 689 on the activity of DNA-directed RNA polymerase is shown in Fig. 1. Addition of this agent at zero time to the RNA polymerase system had essentially no effect on the activity of this enzyme. When the enzyme was incubated with the drug prior to assay of activity, however, a time-dependent inhibition of RNA polymerase activity was observed. Thus, 90% inhibition of enzymatic activity was produced after treatment of RNA polymerase with 1 mM 689 for 60 min. Table 1 shows some of the characteristics of the inhibition of RNA polymerase by 689; inhibition was not antagonized by the addi-

TABLE 1

Characteristics of inhibition of RNA polymerase by 689

RNA polymerase or DNA was incubated with 689 for 60 min and then assayed for activity as described in MATERIALS AND METHODS. In experiment 4, after the initial incubation, the RNA polymerase incubation mixtures (2.0 ml) were dialyzed for 4 hr at 5° against 100 volumes of a mixture of 12.5% DMSO, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 2.5 mM β-mercaptoethanol before assay of RNA polymerase activity. The activity of the "no DNA" controls was subtracted from the experimental values prior to calculation of the percentage decrease from control. Prior incubation of enzyme without drug consistently produced a 10–15% loss of activity as compared with the unincubated enzyme. Therefore, an unincubated control was not included in every experiment. The experiments were not all performed at the same time, and the lower enzymatic activity recorded for experiments 3 and 4 reflects a loss of activity with storage.

Modifications	^{14}C-UTP incorporated	Decrease from control
	μμmoles	%
Experiment 1		
No DNA	17	
Enzyme not initially incubated	1112	
Enzyme incubation		
−Drug	977	
+1 mM 689	108	89
+Bovine serum albumin (50 μg)	1035	
+1 mM 689 and bovine serum albumin (50 μg)	240	78
DNA incubation		
−Drug	1502	
+1 mM 689	1580	
Experiment 2		
No DNA	68	
Enzyme incubation		
−Drug	903	
+1 mM 689	111	95
+1 mM 689 and 3 mM AMP	142	91
+1 mM 689 and 3 mM ATP	167	88
Experiment 3		
No DNA	8	
Enzyme incubation		
−Drug	472	
+1 mM 689	88	83
+0.1 M $MgCl_2$	730	
+1 mM 689 and 0.1 M $MgCl_2$	23	98
Experiment 4		
No DNA	10	
Enzyme incubation		
−Drug	437	
+1 mM 689	83	83
−Drug + dialysis	297	
+1 mM 689 + dialysis	62	82

tion of excess protein (bovine serum albumin), 3 mM ATP, 3 mM AMP, or 0.1 M $MgCl_2$. Dialysis of drug-treated enzyme also failed to restore normal activity, suggesting that 689 must either be tightly bound to the enzyme or produce irreversible changes in the structure of the enzyme.

Contrary to the effect of 689 on RNA polymerase, preliminary incubation of calf thymus DNA with drug did not affect the capacity of DNA to function as a template in the RNA polymerase reaction (experiment 1, Table 1). This observation, in conjunction with the finding that multi-

TABLE 2

Effect of 689 on enzymes involved in synthesis of nucleic acids in E. coli

Enzymes were incubated with 689 for 60 min and then assayed as described in MATERIALS AND METHODS. The amounts of total nucleotides incorporated into RNA and DNA by control samples containing incubated RNA polymerase and DNA polymerase were 4–10 and 4–15 mμmoles, respectively, for the 30-min incubation. Results are presented as the mean percentage inhibition ± standard error as compared to controls where n (number of experi ments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

Concentration of 689	Inhibition	
	RNA polymerase	DNA polymerase
mM	%	%
0.1	11 ± 5.0 (2)	
0.2	24 ± 8.9 (3)	
1.0	81 ± 6.9 (7)	5.3 ± 3.7 (3)
2.0	100 ± 0 (2)	23 ± 5.8 (6)

plication of bacteriophage is inhibited in 689-treated *E. coli* (3), suggests that 689 does not directly act on template DNA.

Table 2 shows a comparison between the sensitivities of partially purified RNA and DNA polymerase to the action of 689. RNA polymerase was considerably more sensitive to this agent than DNA polymerase and was significantly inhibited by a range of concentrations of the purine deoxyribonucleoside analogue which inhibited RNA synthesis *in vivo*. On the other hand, DNA polymerase did not exhibit this correlation between *in vivo* and *in vitro* sensitivities of nucleic acid synthesis to 689.

Effect of 689 on DNA and RNA polymerase activities in bacterial chromosome preparations of E. coli. To be assured that the experiments performed with purified polymerases did not yield results peculiar to that type of system, the effects of 689 on DNA and RNA polymerase activities were examined in two types of chromosomal preparations: (a) a membrane preparation containing both DNA and the polymerase enzymes, and (b) a deoxyribonucleoprotein preparation obtained from the soluble fraction of the cell extract. The latter DNA-protein complex containing polymerase activity is composed of pieces of the bacterial chromosome that have been sheared from the cell membrane during the extraction procedure. The results in Table 3 illustrate that the effects of drug on polymerase activities in the chromosomal preparations were similar to the effects on the partially purified enzymes, in that RNA polymerase was more sensitive to 689 than DNA polymerase. Therefore, a possible molecular site of action of 689 in producing inhibition of the synthesis of both RNA and induced enzyme *in vivo* is RNA polymerase. On the other hand, the insensitivity of DNA polymerase to this agent *in vitro* suggests that another site in the pathway for DNA synthesis is subject to inhibition by 689 *in vivo*, since the drug has a greater effect on DNA synthesis than on RNA synthesis in the growing cell (1–3).

Effects of 689 on other enzymes involved in DNA synthesis. In the search for molecular sites of action responsible for the inhibition by 689 of the synthesis of DNA *in vivo*, several enzymatic pathways involved in the formation of DNA were tested for sensitivity to the drug. Table 4 shows the effect of 689 on the *in vivo* metabolism of ^{14}C-uracil. The conversion of uracil to uridine, cytidine, deoxyuridine, and deoxycytidine nucleotides was inhibited by 689 to approximately the same degree, suggesting (a) an inhibition by the purine deoxyribonucleoside analogue of the first enzymatic reaction (uracil phosphoribosyltransferase, EC 2.4.2.9) or of an early step in the conversion of uracil to other nucleotides, or (b) a generalized suppression of nucleotide metabolism as the result of the blockade of DNA synthesis. In contrast, 689 produced essentially complete blockade of the formation of thymine nucleotides from labeled uracil *in vivo*. Similar results were obtained using ^{14}C-cytosine as a precursor. The effect of 689 on thymine nucleotide synthesis *in vivo* appears to be due to some mechanism other than inhibition of thymidylate synthetase, since thymidylate synthetase activity was not inhibited *in vitro* when the enzyme was incubated with 0.5–2.0 mM 689 for 60 min prior to assay

TABLE 3

Effect of 689 on DNA and RNA synthesis directed by a deoxyribonucleoprotein complex and membrane fraction from E. coli

The deoxyribonucleoprotein complex (DNP) and the membrane preparations were incubated with 689 for 60 min, and the DNA and RNA polymerase activities in these preparations then were determined as described in MATERIALS AND METHODS.

Modifications	^{14}C-TTP incorporated into DNA	Decrease from control	^{14}C-CTP incorporated into RNA	Decrease from control
	μμmoles	%	*μμmoles*	%
Experiment 1				
Zero time control	60		20	
DNP incubated without drug	9760		4930	
DNP incubated with 689				
0.2 mM	9350	4	4380	11
1.5 mM	7180	27	1310	73
			^{14}C-ATP	
Experiment 2				
Zero time control	0		0	
Membrane fraction incubated without drug	817		145	
Membrane fraction incubated with 689				
0.5 mM	705	14	107	26
1.0 mM	667	18	70	52
2.0 mM	585	28	38	74

of conversion of ^{3}H-dUMP to thymidylate.

Effects of 689 on various types of enzymes. The observations of the sensitivity of RNA polymerase to 689 and the relative resistance of DNA polymerase and thymidylate synthetase to the drug prompted further experiments to determine what types of enzymes are affected by this purine deoxyribonucleoside analogue. Table 5 lists the effects of 689 on a variety of enzymes which degrade nucleic acids, nucleotides, and protein. DNase and RNase from bovine pancreas and polynucleotide phosphorylase from *E. coli* B were sensitive to the drug, whereas trypsin (bovine pancreas) and *E. coli* alkaline phosphatase were resistant.

Differential effects of 689 on protein synthesis in vitro directed by endogenous E. coli mRNA or by synthetic polyribonucleotides. The addition of 689 at zero time to an *in vitro* protein-synthesizing system (S-30) from *E. coli* resulted in little effect on the incorporation of ^{14}C-amino acids into protein. However, as demonstrated with RNA polymerase, preliminary incubation of the S-30 fraction with 689 produced an inhibition of protein synthesis. The effect

TABLE 4

Effect of 689 on metabolism of uracil-2-^{14}C in vivo

6-Choro-8-aza-9-cyclopentylpurine (0.5 mM) and uracil-2-^{14}C were added to a growing culture of *E. coli* B. After 3 min, the cells were extracted and the soluble fraction was analyzed as described in MATERIALS AND METHODS. Results are expressed as the percentage of the radioactivity incorporated into control cells not treated with drug.

Nucleoside formed	Percentage of control
Uridine	52
Cytidine	37
Deoxyuridine	50
Deoxycytidine	41
Thymidine	4

TABLE 5

Effect of 689 on enzymes that degrade nucleic acid and protein

Enzymes were incubated with 689 for 60 min and then assayed as described in MATERIALS AND METHODS. Control-incubated DNase, RNase, and alkaline phosphatase had activities of 45–60 units, 5–10 units, and 0.065 unit/5 μg of protein, respectively. Incorporation of ^{14}C-ADP into RNA by control-incubated polynucleotide phosphorylase was approximately 100 mμmoles/reaction/15 min. Unincubated trypsin had an activity of 36 units/10 μg of protein; the control enzyme activities after incubation for 60 min at 37° and 25° were 2 units and 22 units/10 μg of protein, respectively. The presence of 2 mM 689 in the preliminary incubation media stimulated the activity of trypsin at both 25° and 37° (37–40 units/10 μg) as compared to the incubated controls. Results are presented as the mean percentage inhibition from control ± standard error where n (number of experiments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

Concentration of drug	Inhibition				
	DNase I	RNase B	Trypsin	Alkaline phosphatase	Polynucleotide phosphorylase
mM	%	%	%	%	%
0.2	14 ± 5 (2)	43 ± 7 (2)		0 (1)	17 ± 6 (2)
1.0	34 ± 6 (2)	72.3 ± 10.7 (3)		0 (1)	46 ± 6 (2)
2.0	78.2 ± 9.0 (4)	97.0 ± 3.0 (3)	0 ± 0 (2)	0 (1)	83 (1)

of a 60-min incubation of S-30 with 689 on protein synthesis *in vitro* using endogenous messenger RNA is shown in Table 6. It was observed that 689 produced a differential inhibition of ^{14}C-labeled phenylalanine, proline, and lysine incorporation into protein.

It had previously been noted that 689 (0.4 mM) inhibited protein synthesis in *E. coli* B (3); however, since the drug also inhibited the formation of RNA, it was possible that the concurrent blockade of protein synthesis was simply a reflection of the inhibition of RNA synthesis. The

TABLE 6

Differential effects of 689 on incorporation of various amino acids into protein directed by endogenous E. coli mRNA or synthetic polynucleotides

S-30 was incubated with 689 for 60 min, after which incorporation of amino acids into protein was determined as described in MATERIALS AND METHODS. In experiments utilizing endogenous mRNA, control values for incorporation of ^{14}C-labeled phenylalanine, proline, and lysine were approximately 1200–1400 cpm, 280–600 cpm, and 500–600 cpm, respectively, per reaction mixture. Samples deproteinized at zero time were also used as controls in each experiment. In experiments utilizing synthetic mRNAs, control values for incorporation of ^{14}C-phenylalanine, ^{14}C-lysine, and ^{14}C-proline were 4000–25,000 cpm, 6000–18,000 cpm, and 4000–12,000 cpm, respectively, per reaction mixture. A reaction mixture containing no polymer was also used as a control in each experiment in order to determine endogenous *E. coli* mRNA activity. This residual activity was subtracted from total radioactivity to give a measurement of the coding capacity of each synthetic polyribonucleotide. Results are expressed as the mean percentage change from control ± standard error where n (number of experiments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

^{14}C-Amino acid	mRNA	Change from control		
		0.5 mM 689	1.0 mM 689	2.0 mM 689
		%	%	%
Phenylalanine	Endogenous	+24.8 ± 17.0 (5)	+15.1 ± 7.6 (6)	−4.2 ± 4.6 (5)
Lysine	Endogenous	−24.5 ± 5.2 (6)	−36.4 ± 7.7 (6)	−81.2 ± 8.5 (5)
Proline	Endogenous	−15.5 ± 5.8 (4)	−37.0 ± 4.5 (4)	−49.8 ± 6.9 (4)
Phenylalanine	Poly U	+22.4 ± 9.8 (5)	+48.7 ± 16.5 (4)	+74.3 ± 35.2 (6)
Lysine	Poly A	−11.0 ± 4.4 (3)	−14.0 ± 2.1 (3)	−75.3 ± 5.2 (3)
Proline	Poly C	−9 (1)	−31 (1)	−85 (1)

data in Table 6 indicate that protein synthesis *in vitro* can be inhibited directly by the same concentration range of 689 that is effective *in vivo*.

Table 6 also shows the results obtained when the S-30 fraction (minus amino acids, ATP-generating system, and polynucleotide) was treated with 689 before examination of polypeptide-synthesizing capacity as directed by synthetic polyribonucleotides. A differential effect on polypeptide synthesis, which depended on the synthetic polymer employed as messenger RNA, was observed. The syntheses of both poly A-directed ^{14}C-polylysine and poly C-directed ^{14}C-polyproline were inhibited. Poly U-directed peptide synthesis was either unchanged or stimulated, but inhibition was not observed in any of the experiments.

Effect of 689 on ribosomal structure and function in protein synthesis. To investigate further the mechanism of action of 689 on *in vitro* protein synthesis, the effect of 689 on ribosomal structure and function was examined. Figure 2 shows the sedimentation profiles of 30 S, 50 S, and 70 S ribosomes obtained from *E. coli* cells exposed to 0.4 mM 689. No change in sedimentation coefficient or profile was noted, indicating that 689 had no apparent effect on ribosomal structure.

The function of ribosomes after incubation for 30 min with 689 was tested by measuring their capacity for polypeptide synthesis as directed by synthetic polymers. The results of these experiments (Table 7) indicate that the ability of the ribosomes to function in protein synthesis was only slightly affected by 689. Again, the incorporation of phenylalanine into protein appeared to be stimulated by 689.

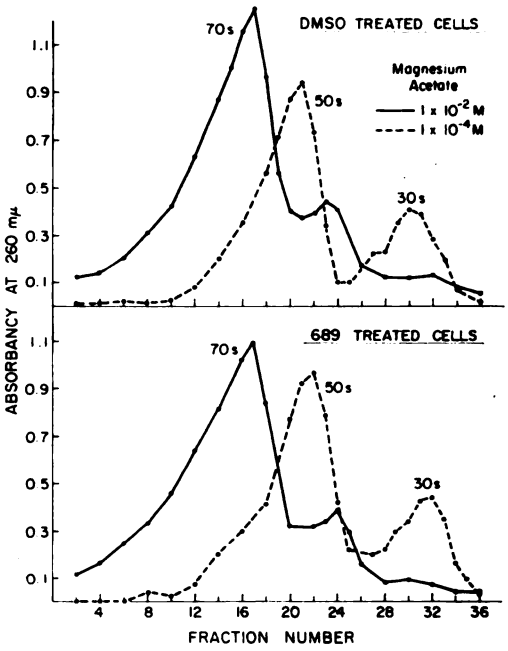

FIG. 2. *Effect of 689 on the structural integrity of E. coli ribosomes in vivo*

E. coli B cells growing in minimal salts media (in log phase) were treated with 0.4 mM 689 for 15 min. Ribosomes were prepared from these cells and subjected to sucrose density gradient centrifugation as described in MATERIALS AND METHODS.

The observation that 689 produced differ-

TABLE 7

Effect of 689 on capacity of E. coli ribosomes to synthesize polypeptide

Washed ribosomes were incubated with 689 for 30 min and then assayed for activity by measuring synthetic polyribonucleotide-directed ^{14}C-amino acid incorporation into polypeptides as described in MATERIALS AND METHODS and the legend to Table 6. Results are expressed as the mean percentage change from control ± standard error where n (number of experiments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

^{14}C-Amino acid	Polymer	Change from control			
		0.5 mM 689	1.0 mM 689	2.0 mM 689	3.0 mM 689
		%	%	%	%
Phenylalanine	Poly U	+6.5 ± 0.5 (4)	+11.2 ± 3.7 (5)	+20.6 ± 4.2 (8)	0 (2)
Lysine	Poly A	0 (2)	0 (2)	0 (2)	−33.0 ± 4.4 (3)
Proline	Poly C	0 (2)	−10.0 ± 1.0 (3)	−12.2 ± 1.7 (5)	

ential effects on polypeptide synthesis prompted an examination of the binding of ^{3}H-poly U to 689-treated ribosomes. *E. coli* ribosomes obtained from cells treated for 15 min with 0.4 mM 689 bound ^{3}H-poly U to a greater extent than control ribosomes (Fig. 3). Ribosomes treated *in vitro* with 689 also bound ^{3}H-poly U to a greater extent than controls. In contrast, the binding of ^{3}H-poly A to ribosomes was not affected by 689. Therefore, the small stimulation of poly U-directed ^{14}C-polyphenylalanine synthesis by 689 may be the result of increased binding of poly U to the ribosomes.

The effects of 689 on ribosomal activity are small in comparison to the effects on protein synthesis in the complete S-30 fraction. It seems likely, therefore, that the effects of this agent on ribosomes *in vivo* are not of great significance in the mechanism of inhibition of protein synthesis.

Effect of 689 on activity of S-100 fraction in polypeptide synthesis. The results obtained by incubating the S-100 (100,000 $\times g$ supernatant) fraction with various concentrations of 689 for 30 min are shown in Table 8. The inhibition of S-100 activity in directing polypeptide synthesis was similar to that observed when the S-30 fraction had been treated with 689 (Table 6). Inhibition of S-100 activity *in vitro* by 689 was attained with a concentration range of drug which inhibited protein synthesis in the whole cell. The kinetics of this inhibition is shown in Table 9. After 30 min of incubation with the purine deoxyribonucleoside analogue, the activity of S-100 in catalyzing poly C-directed ^{14}C-polyproline synthesis was essentially abolished, while activity in poly A-directed ^{14}C-polylysine synthesis was inhibited approximately 50%. Experiment 2 in Table 9 shows that dialysis of the S-100 fraction did not restore activity, indicating either relatively tight

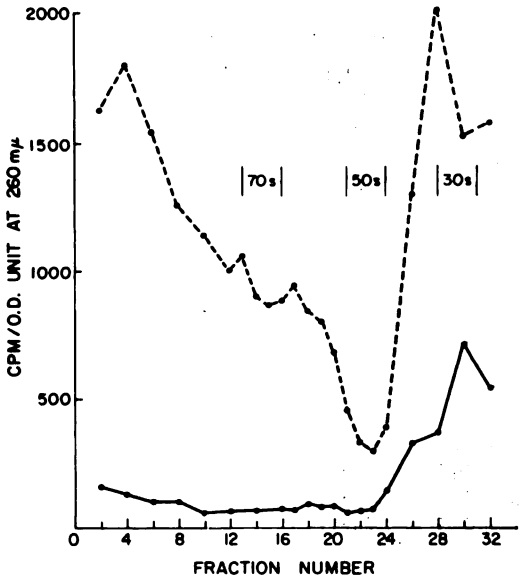

FIG. 3. *Effect of 689 on the binding of ^{3}H-poly U to E. coli B ribosomes*

^{3}H-Poly U was added to a ribosomal suspension extracted from an *E. coli* B culture which had been exposed to 689 (0.4 mM) for 15 min. Binding of ^{3}H-poly U to ribosomes was measured by density gradient centrifugation as described in MATERIALS AND METHODS. ●——●, DMSO control; ● - - - ●, 689-treated.

TABLE 8

Effect of 689 on protein-synthesizing enzymes present in S-100 fraction of E. coli

S-100 was incubated with 689 for 30 min and then assayed for activity by measuring synthetic polyribonucleotide-directed ^{14}C-amino acid incorporation into polypeptides as described in MATERIALS AND METHODS and the legend to Table 6. Results are expressed as the mean percentage change from control ± standard error where n (number of experiments enclosed in parentheses) exceeds 2, and as ± range where $n = 2$.

^{14}C-Amino acid	Polymer	Change from control			
		0.5 mM 689	1.0 mM 689	2.0 mM 689	3.0 mM 689
		%	%	%	%
Phenylalanine	Poly U	+28.0 ± 21.0 (4)	+40.5 ± 22.3 (4)	+32.7 ± 20.7 (7)	+13 (1)
Lysine	Poly A	+9.0 ± 2.0 (2)	0 (3)	−40.4 ± 8.2 (5)	−76.5 ± 6.5 (2)
Proline	Poly C	−20.5 ± 6.7 (4)	−54.2 ± 7.5 (4)	−90.3 ± 3.3 (6)	

TABLE 9

Kinetics and irreversibility of the inhibition by 689 of S-100 polypeptide-synthesizing activity

In experiment 1, S-100 was incubated with 2 mM 689 and then assayed for activity by measuring synthetic polyribonucleotide-directed ^{14}C-amino acid incorporation into polypeptides as described in MATERIALS AND METHODS. In experiment 2, S-100 was incubated with 2 mM 689 for 30 min, and the incubation mixtures (2.0 ml) were dialyzed for 4 hr at 5° against 100 volumes of a solution containing 12.5% DMSO, 15 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 30 mM KCl, and 3 mM β-mercaptoethanol before assay of S-100 activity. Dialysis was performed only on the samples marked "+ dialysis."

Modifications	Poly C-directed ^{14}C-proline incorporation		Poly A-directed ^{14}C-lysine incorporation		^{14}C-proline incorporation
	−689	+2 mM 689	−689	+2 mM 689	
	cpm		*cpm*		*cpm*
Experiment 1					
No preliminary incubation	11,125	8,744	20,470	18,330	
15-min incubation	11,047	1,794	20,349	16,301	
30-min incubation	11,051	978	23,263	12,295	
60-min incubation	11,353	683	19,414	10,713	
Experiment 2					
No poly C					752
S-100 preliminary incubation					
−Drug					5,959
+Drug					812
−Drug, +dialysis					7,796
+Drug, +dialysis					636

binding or irreversible changes which decreased the activity of some component of the polypeptide-synthesizing system.

Two of the enzymatic functions present in the S-100 fraction that are presently known to participate in polypeptide synthesis are aminoacyl-tRNA synthetase and soluble transfer factors (22). The effects of 689 on aminoacyl-tRNA synthetase activity were studied by incubating S-100 with drug and then measuring the binding of the ^{14}C-labeled amino acids phenylalanine, proline, lysine, leucine, and valine to tRNA. The results of these experiments indicated that none of the synthetase enzymes was sensitive to 689. Thus, the inhibition of *in vitro* protein synthesis by this drug does not appear to be due to inhibition of aminoacyl-tRNA synthetases and may reflect an action of the drug on transfer factors. The lack of a significant effect of 689 on the function of ribosomes in protein synthesis seems to rule out peptide synthetase, a ribosome-associated enzymatic activity (23), as a site of drug action. It also seems unlikely that 689 inhibits protein formation *in vivo* by affecting ribosomal factors involved in the initiation of protein synthesis (24), since a marked depression of polypeptide synthesis was observed in the *in vitro* system directed by synthetic polynucleotides, where initiation factors are apparently not a requirement.

DISCUSSION

The structure of 6-chloro-8-aza-9-cyclopentylpurine suggests that this purine deoxyribonucleoside analogue could affect cells in several different ways. The presence of a chlorine atom in position 6 of the azapurine portion of the molecule may confer on this agent the capacity to inhibit nucleotide metabolism in a manner analogous to the blockade of the conversion of inosine 5′-phosphate to guanosine 5′-phosphate by 6-chloropurine (25, 26). Moreover, the related derivative, 9-cyclopentyladenine, has been found to be capable of decreasing the rate of formation of 5-phosphoribosyl 1-pyrophosphate (27), a substrate required in the synthesis of both purine and pyrimidine nucleotides. The relatively uniform

inhibition by 689 of the conversion of uracil and cytosine *in vivo* to the nucleotides or uridine, cytidine, deoxycytidine, and deoxyuridine could be the result of a blockade of the synthesis of 5-phosphoribosyl 1-pyrophosphate.

The cyclopentyl ring portion of 689, which cannot be phosphorylated, prevents the compound from being incorporated into DNA and RNA; therefore, barring cleavage to the base, 689 is incapable of altering the function of nucleic acids in a manner analogous to 8-azaguanine, which is incorporated into and alters the function of RNA (28–30), and 7-deazaadenosine (tubercidin), which is incorporated into DNA (31).

The observation that 689 decreased by approximately 50–60% the conversion of uracil and cytosine to nucleotide metabolites other than the thymine nucleotides, while producing almost complete inhibition of the formation of thymine nucleotides (Table 4), suggests a major site of drug action in the pathway involved in synthesizing nucleotide derivatives of thymine. *In vitro* studies, however, demonstrated that the drug had no effect on thymidylate synthetase activity. It is conceivable, therefore, that inhibition of thymidylate formation by 689 is due to interference with the synthesis of N^5,N^{10}-methylenetetrahydrofolate, an essential coenzyme in the conversion of dUMP to TMP. The pronounced inhibition of the formation of thymine nucleotides by this agent could explain the greater effect of 689 on DNA synthesis than on RNA synthesis noted *in vivo* (1–3), since the differential effect of this agent on nucleic acid synthesis cannot be accounted for by inhibition of DNA polymerase.

It had been suggested previously that the chlorine atom in position 6 of the purine nucleus of 689 could make the compound a potent "alkylating agent" (1). Moreover, the presence of the electropositive cyclopentyl ring and 8-aza group in 689 renders the 6-chloro atom more labile than it is in 6-chloropurine 2′-deoxynucleoside and could produce a carbonium ion at position 6 of the purine analogue. It is conceivable, therefore, that the drug inhibits RNA polymerase, as well as other sensitive enzymes, by being directed into an active site of the enzyme as a result of its "nucleoside" structure and then irreversibly binding to the enzyme by alkylating electronegative groups of the protein. The observations that 689 does, indeed, irreversibly inhibit RNA polymerase activity *in vitro,* and that substitution of the chlorine atom at position 6 with either hydroxyl, amino, or hydrazino (i.e., less ionizable groups) lessens the effect of the drug on induced enzyme synthesis (1) and on the inhibition of bacteriophage replication (32), support the concept that this agent functions, in part, by an alkylation mechanism. However, the drug does not appear to alkylate nucleophilic centers at random. DNA template activity, for instance, was not affected by 689 even after incubation of DNA with drug for up to 16 hr. Likewise, 689 had little effect on enzymatic proteins such as trypsin, alkaline phosphatase, or DNA polymerase (Table 5), and bovine serum albumin did not antagonize the drug-induced inhibition of RNA polymerase activity (Table 1). It is possible that alkylation of these proteins occurs, but that unless 689 binds to the active site of an enzyme, a negligible effect on enzymatic function is achieved. The fact that 689 is effective *in vitro* against enzymes which either polymerize or degrade nucleic acid molecules suggests that the purine deoxyribonucleoside structure of 689 allows for a site-directed alkylation of receptors with ensuing blockade of enzymatic activity.

Observations *in vivo* (1–3) have shown that (a) 689 produces essentially an immediate effect on the syntheses of thymidylate and DNA (within 3 min), and (b) the effects of the drug on the formation of RNA and protein are less potent and are dissociated from the blockade of DNA biosynthesis. *In vitro,* RNA polymerase was not immediately inhibited by 689, but required a period of incubation with the drug. It is interesting that the bactericidal action of 689 on *E. coli* cells depends on the length of time the cells are in contact with

drug (3), and this time period appears to correlate well with the time required for 689 to inhibit completely RNA polymerase *in vitro* (Fig. 1).

The agent 689 appears to exert a direct action on protein synthesis *in vitro*, possibly through an action on soluble transfer factors. It appears that the soluble transfer factors (T factors) of *E. coli* have a function similar to, if not identical with, that of the transfer factor (transferase I) described for the rat liver system (22, 33). It has been demonstrated recently (33) that transferase I of rat liver can be inhibited by GTP, all ribonucleoside diphosphates, and 5′-guanyl methylenediphosphonate if the enzyme has been incubated for at least 10 min with the nucleotides in the absence of aminoacyl-tRNA (the presence of aminoacyl-tRNA prevents the loss of enzyme activity). These results may reflect a control mechanism for the regulation of transferase I activity by nucleotides. The inhibition of polypeptide synthesis by 689 in *E. coli* B extracts (S-100) also required prior incubation of the drug with the enzyme preparation, suggesting that this agent may inhibit transferase I activity by a similar mechanism. Dialysis of 689-treated S-100 extracts did not restore the capacity to fabricate polypeptides. Ibuki and Moldave (33) have demonstrated that nucleotide-induced loss of transferase I activity was also irreversible.

From the above observations, it can be seen that the purine deoxyribonucleoside analogue 689 apparently has some unique actions on various biosynthetic pathways and therefore may prove to be a useful tool in unraveling the molecular events involved in nucleic acid and protein synthesis.

ACKNOWLEDGMENTS

The authors wish to thank Miss Linda Galligher and Miss Bonnie Wachter for their excellent technical assistance.

REFERENCES

1. M. S. Zedeck, A. C. Sartorelli, P. K. Chang, K. Raška, Jr., R. K. Robins and A. D. Welch, *Mol. Pharmacol.* **3**, 386 (1967).
2. M. S. Zedeck, A. C. Sartorelli, J. M. Johnson and R. W. Ruddon, *Bacteriol. Proc.* **32** (1968).
3. M. S. Zedeck, A. C. Sartorelli, J. M. Johnson and R. W. Ruddon, *Mol. Pharmacol.* **5**, 263 (1969).
4. A. Tissières, J. D. Watson, D. Schlessinger and B. R. Hollingworth, *J. Mol. Biol.* **1**, 221 (1959).
5. D. B. Cowie, G. N. Cohen, E. T. Bolton and H. de Robichon-Szulmajster, *Biochim. Biophys. Acta* **34**, 39 (1959).
6. M. W. Nirenberg and J. H. Matthaei, *Proc. Nat. Acad. Sci. U. S. A.* **47**, 1588 (1961).
7. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
8. D. H. Shin and K. Moldave, *J. Mol. Biol.* **21**, 231 (1966).
9. K. Burton, *Biochem. J.* **62**, 315 (1956).
10. R. W. Ruddon and J. M. Johnson, *Mol. Pharmacol.* **4**, 258 (1968).
11. R. J. Crouch, Bacteriophage T4 ribonucleic acid synthesis, *in vivo* and *in vitro*. Ph.D. thesis, University of Illinois, 1968.
12. C. C. Richardson, C. L. Schildkraut, H. V. Aposhian and A. Kornberg, *J. Biol. Chem.* **239**, 222 (1964).
13. M. Chamberlin and P. Berg, *Proc. Nat. Acad. Sci. U. S. A.* **48**, 81 (1962).
14. F. R. Williams and M. Grunberg-Manago, *Biochim. Biophys. Acta* **89**, 66 (1964).
15. M. I. Smith Lomax and G. R. Greenberg, *J. Biol. Chem.* **242**, 109 (1967).
16. M. Kunitz, *J. Gen. Physiol.* **33**, 349 (1950).
17. G. Kalnitsky, J. P. Hummel and C. Dierks, *J. Biol. Chem.* **234**, 1512 (1959).
18. B. C. W. Hummel, *Can. J. Biochem. Physiol.* **37**, 1393 (1959).
19. A. Garen and C. Levinthal, *Biochim. Biophys. Acta* **38**, 470 (1960).
20. J. M. Johnson and R. W. Ruddon, *Mol. Pharmacol.* **3**, 195 (1967).
21. G. Suarez and D. Nathans, *Biochem. Biophys. Res. Commun.* **18**, 743 (1965).
22. R. Ertel, N. Brot, B. Redfield, J. E. Allende and H. Weissbach, *Proc. Nat. Acad. Sci. U. S. A.* **59**, 861 (1968).
23. R. E. Monro, *J. Mol. Biol.* **26**, 147 (1967).
24. W. M. Stanley, Jr., M. Salas, A. J. Wahba and S. Ochoa, *Proc. Nat. Acad. Sci. U. S. A.* **56**, 290 (1966).
25. A. C. Sartorelli and B. A. Booth, *J. Pharmacol. Exp. Ther.* **134**, 123 (1961).
26. A. C. Sartorelli and B. A. Booth, *Biochim. Biophys. Acta* **55**, 214 (1962).
27. G. Tyrsted and A. C. Sartorelli, *Biochim. Biophys. Acta* **155**, 619 (1968).

28. I. Lasnitski, R. E. F. Matthews and J. D. Smith, *Nature* **173**, 346 (1954).
29. H. G. Mandel, *Pharmacol. Rev.* **11**, 743 (1959).
30. E. F. Zimmerman, B. W. Holler and G. D. Pearson, *Biochim. Biophys. Acta* **134**, 402 (1967).
31. G. Acs, E. Reich and M. Mori, *Proc. Nat. Acad. Sci. U. S. A.* **52**, 493 (1964).
32. P. K. Chang, L. J. Sciarini, A. C. Sartorelli and M. S. Zedeck, *J. Med. Chem.* **11**, 513 (1968).
33. F. Ibuki and K. Moldave, *J. Biol. Chem.* **243**, 44 (1968).